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Unc13 Aligns SNAREs and Superprimes Synaptic Vesicles

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Unc13 proteins are required for vesicle docking and priming during exocytosis. In this issue of *Neuron*, Lai et al. (2017) demonstrate that Unc13 ensures that the SNAREs assemble into functional subcomplexes. In a second manuscript, Michelassi et al. (2017) identify a previously unknown autoinhibited state for Unc13 mediated by the tandem C1 and C2 domains.

Fusion of synaptic vesicles with the plasma membrane is mediated by the SNARE proteins syntaxin, SNAP-25, and synaptobrevin. In addition to the SNAREs, functional studies demonstrate that two families of synaptic proteins have nearly as profound a role in fusion as the SNARE proteins: Unc18/Munc18 proteins and the Unc13/Munc13 family of proteins. In vivo elimination of Unc18 or Unc13 comes close to fully eliminating fusion. Like the SNAREs, a homolog of Unc18 (known as SM proteins) is required for all SNARE-mediated fusions in the cell. On the other hand, the Unc13 family appears to be specialized for calcium-triggered synaptic vesicle release. The Unc13 family is of particular interest since it sits at an important hub: it directly regulates SNAREs and is also the target of modulators of synaptic activity. In anticipation of exocytosis, synaptic vesicles become physically anchored to the plasma membrane, where they undergo a second step that makes them fusion competent. These steps are known as docking and priming, respectively. Primed vesicles can also be put into an enhanced highly fusogenic state called superpriming. Unc13 has been proposed to act at each of these steps: docking, priming, and superpriming. All Unc13 family members are composed of a diacylglycerol-binding domain (C1 domain), a calcium and phospholipid-binding domain (C2 domain), and a conserved domain found in Unc13 family members (MUN domain) that is composed of stacked alpha helices (Figure 1) (Xu et al., 2017). In this issue of *Neuron*, two complementary papers expand our understanding of Unc13. Lai et al. (2017) demonstrate that Unc13 orients and registers SNAREs during molecular priming of vesicles, and Michelassi et al. (2017) identify the C1-C2 module as an autoinhibitory domain that is disinhibited by diacylglycerol (DAG) or calcium. The disinhibition step likely allows for the superpriming of synaptic vesicles.

One of the first clues that Unc13 regulates SNARE formation came from the observation that a constitutively open form of syntaxin could bypass the requirement for Unc13 in vesicle fusion (Richmond et al., 2001), suggesting that the function of Unc13 is to open syntaxin during SNARE complex formation. Biochemical assays demonstrated that the MUN domain is necessary and largely sufficient for this activity (Ma et al., 2011). This is now known to be the first step in SNARE complex formation (see Figure 1). In Unc13 mutants, there is a decrease in docked vesicles, and the defect in docking is completely suppressed by expression of open syntaxin (Hammarlund et al., 2007). However, loss of Unc13 can only be partially bypassed by open syntaxin; evoked responses are greatly reduced, suggesting that these docked vesicles are not fusion competent. There must be additional roles for Unc13.

Lai et al. (2017) use fluorescence resonance energy transfer (FRET) assays and tethered vesicle fusion assays to demonstrate that Unc13 orients SNARE proteins during pairing. To fuse membranes, the SNAREs must nucleate assembly at their N termini and zipper toward their C termini anchored in the membrane—that is, they must be aligned in a parallel configuration (Sutton et al., 1998). Assembly in this configuration is not guaranteed, and previously the Brunger lab demonstrated that SNAREs can also form dead-end antiparallel configurations in vitro (Weninger et al., 2003).

In the current manuscript, Lai et al. (2017) find that, in biochemical assays, open syntaxin readily forms SNARE complexes with synaptobrevin and SNAP25; however, at least half of these complexes have incorporated anti-parallel helices, even in the presence of Unc18. The formation of these dead-end SNARE complexes explains why in vivo the open form of syntaxin can bypass Unc13 to produce morphologically docked vesicles but cannot rescue fusion. By contrast, when Lai et al. (2017) included both the Unc13 MUN domain and Unc18 in their assay, correctly oriented syntaxin-SNAP25 complexes were observed. Thus, in this second step, both Unc13 and Unc18 are required to assemble a functional target-SNARE (t-SNARE) complex.

To identify further roles for Unc13, Lai et al. (2017) started with an assembled syntaxin and SNAP25 t-SNARE complex. Upon addition of synaptobrevin, SNARE complexes freely formed, but once again, only about half were in the correct orientation. The addition of the MUN domain alone ensured that synaptobrevin was incorporated in a parallel orientation with 95% efficiency. These results indicate that, in this third step, Unc13 alone is responsible for registering and incorporating synaptobrevin into the complete SNARE complex.

Does SNARE assembly by Unc13 support calcium-dependent membrane fusion? To answer this question, Lai et al. (2017) tested the role of Unc13 in an in vitro fusion assay. In this assay,

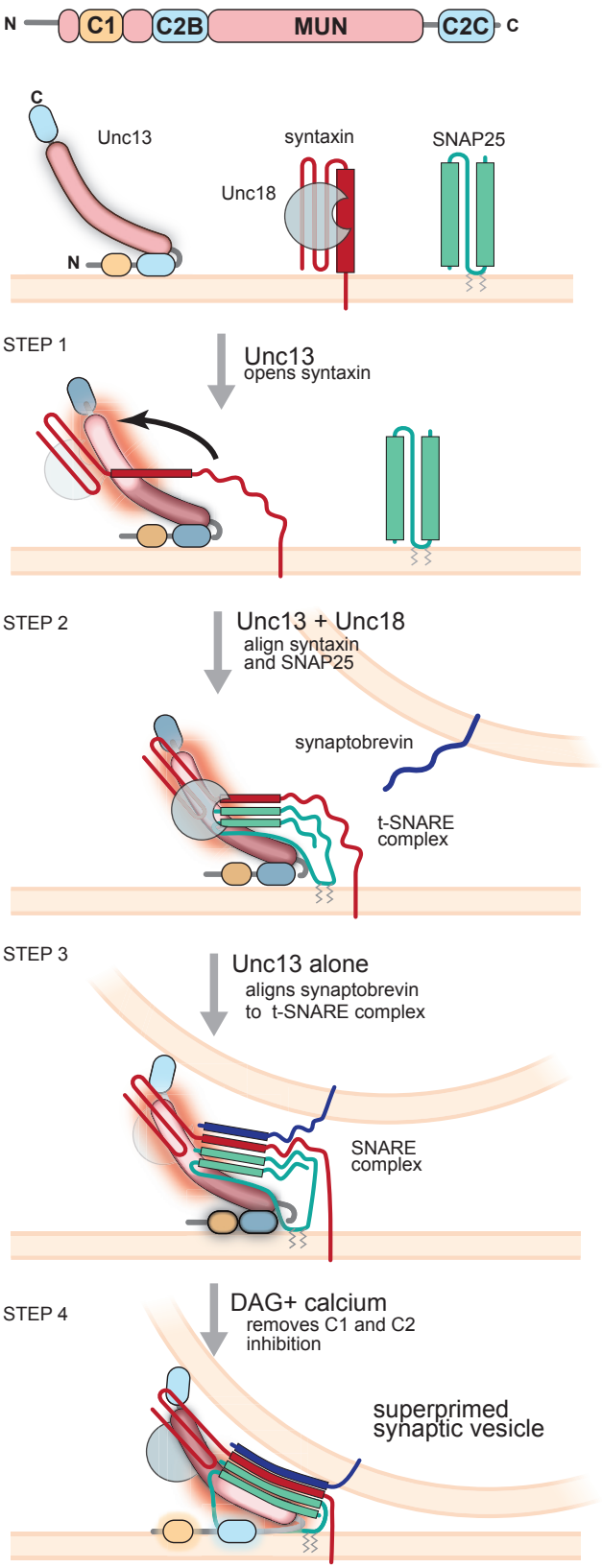

Figure 1. Priming of Synaptic Vesicles by Unc13

Unc13 proteins share a common structure composed of C1, C2, and a MUN domain. The MUN domain is assembled into stacked alpha helices (pink), some of which precede or are interspersed with the C1 and C2 domains (Xu et al., 2017). In step 1, Unc13 firsts interacts with the syntaxin-Unc18 and converts syntaxin to the open configuration. In step 2, Unc13 and Unc18 together align and assemble the t-SNARE complex. In step 3, Unc13 alone is able to properly align and incorporate synaptobrevin into the SNARE complex, and the vesicle is now in a molecularly primed state. In step 4, DAG and calcium binding to the C1-C2 module disinhibits the MUN domain and increases the sensitivity of the primed vesicle for calcium. How the fusion complex is altered is not known. It is possible that it is simply physical propinquity: the synaptic vesicle is simply drawn closer to the plasma membrane. Alternatively, there is an allosteric change in the fusion complex, either in the MUN domain of Unc13, in the calcium sensor, or in SNARE assembly (symbolized as a tighter association of the SNARE proteins and close association of the vesicle with the plasma membrane).

vesicles containing the reconstituted t-SNARE complex are tethered to a glass coverslip; v-SNARE vesicles are added and fusion is monitored in a fluorescence assay using total internal reflection fluorescence (TIRF). When the v-SNARE vesicles include the calcium sensor proteins synaptotagmin and complexin, calcium-dependent fusion is observed when tested with a high concentration of calcium (500 μM), thereby recapitulating calcium-dependent fusion of membranes. Inclusion of the MUN domain (but not Unc18) in the assay tripled membrane fusion in response to high-concentration calcium. When the C1-C2B-MUN fragment or the complete C1-C2B-MUN-C2C were included, fusion efficiency was greatly increased, and 20-fold and 100-fold less protein were required to achieve the same level of fusion, respectively. Importantly, addition of Unc13 in these assays reduced the half maximal responses to calcium to 80 μM, close to values observed in vivo.

When the t-SNARE vesicles were reconstituted with Unc18 and the C1-C2B-MUN construct, a physiological calcium sensitivity of 23 μM was achieved, thereby essentially reconstituting synaptic vesicle fusion in vitro. Interestingly, after SNARE assembly, the MUN domain is dispensable and could be washed out of the prep, and the docked vesicles maintained calcium-dependent fusion.

However, in vivo it is unlikely that Unc13 departs from the docking site, given its known interactions with other active zone components and given its role in superpriming (see below).

The Unc13 C1-C2B module is the major target of regulation by second messengers at synapses. The C1 domain binds the lipid DAG, which is produced by activating phospholipase C. The C2B domain is regulated by the binding of calcium and the phospholipid PIP2. But how do DAG and calcium regulate Unc13? Previous studies have suggested that the C1-C2B module acts as a *positive* effector of vesicle fusion (Betz et al., 1998; Rhee et al., 2002). By contrast, Michelassi et al. (2017) demonstrate that the C1-C2B module is a *negative* regulator of vesicle fusion. They argue that DAG and calcium relieve Unc13 from an autoinhibited state and that this disinhibition occurs after SNARE formation. These data are particularly interesting since they come at the same time as the publication of the structure of the C1-C2B-MUN fragment (Xu et al., 2017).

Early data demonstrated that the C1 domain could recruit Unc13 to the membrane, and it was suggested that this was the mechanism of potentiation of the synapse by DAG. According to these models, increased recruitment of Unc13 to the active zone would generate more syntaxin in the open state and in turn dock more synaptic vesicles. While recruitment of Unc13 to the active zone is clearly essential for its function, there is a substantial fraction of Unc13 already at the membrane, stabilized through its interaction with numerous active zone proteins.

Recently, evidence has emerged that the C1-C2B module might act during, or perhaps more likely after, assembly of the SNARES. Specifically, Unc13 can promote a ''superprimed'' state of docked synaptic vesicles (Ishiyama et al., 2014; Taschenberger et al., 2016). Most primed synaptic vesicles have a low probability of fusion (~10%). However, DAG mimics and strong stimulation protocols, which elevate calcium levels, cause a population of synaptic vesicles to adopt a high probability of fusion (~50%) (Taschenberger et al., 2016). Superpriming might act on already primed vesicles (as illustrated in Figure 1) or might occur during the assembly of SNAREs, for example, by the recruitment of additional factors.

By engineering Unc13 variants into *C. elegans*, the Dittman laboratory demonstrates that the C1-C2B module inhibits MUN domain function, and it is relief from inhibition that allows synaptic vesicles to move into the superprimed state (Michelassi et al., 2017). First, they demonstrated that the C2B domain inhibits Unc13 function. When the C2B domain was deleted, they observed an increase in neurotransmission and a normal response to hypertonic solution—the hallmarks of superprimed vesicles. Moreover, substitution of the C2B domain with a variant that mimicked calcium binding also disinhibited Unc13. Similarly, when Michelassi et al. (2017) deleted the entire C1 domain, synaptic release was increased. Interestingly, either deletion of the C1 domain or the addition of DAG mimics reversed the inhibition imposed by the calcium-binding mutant C2B domain. Together, these data suggest that the C1-C2B module may act as an ''OR'' gate: either the presence of DAG or calcium-binding by the C2B domain can disinhibit Unc13 and increase the probability of release.

Identification of the C1-C2B as an inhibitory module is particularly compelling given the publication of the C1-C2B-MUN structure (Xu et al., 2017). The C2B domain is tightly coupled to the MUN domain in the crystal structure, and this coupling is required for inhibition. First, when Michelassi et al. (2017) added flexibility to the linker between C2B and the MUN domain, they observed an increase in neurotransmission and relief from the inhibited state imposed by a C2B calcium-binding mutant. Second, when a point mutation linked to autism (Lipstein et al., 2017) was engineered into Unc13, neurotransmission was again increased, and inhibition by the C2B domain was relieved. This point mutation is at the tip of a helix in a contact interface with the C2B domain. Together, these linker modifications argue that decoupling C2B from the MUN domain relieves the inhibited state.

The structure of the C1-C2B-MUN also further narrows models for the mechanism of the C1-C2B module (Xu et al., 2017). For instance, the interaction surface of the C1-C2B module with the MUN domain is far from the known syntaxin interaction surface. The structure should represent the inhibited state. Thus, it is unlikely that the C1-C2B module acts by inhibiting SNARE priming, which is consistent with a late role for this regulatory step. The C1-C2B module is buried at the base of a continuous stack of alpha helices. It is, at present, difficult to imagine how calcium and DAG binding will affect this structure. Further progress will probably require a structural study of Unc13 in the fully activated state, as well as characterizing how Unc13 interacts with the fully assembled fusion complex.

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